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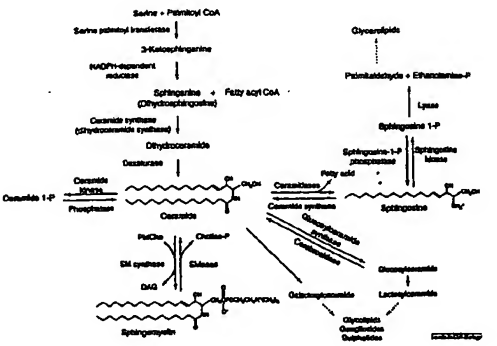

(57) Abstract: The present invention relates to ceramidase genes, in particular to human mitochondrial ceramidase genes and their encoded protein products, as well as derivatives and analogs thereof. Production of ceramidase proteins, derivatives, and antibodies are also provided. The present invention relates to methods for treating and preventing hyperproliferative diseases, cardiovascular diseases and inflammation based on the regulation of the level of ceramide. In particular, the invention relates to the regulation of the level of ceramide by inhibiting ceramidase expression or activity. The invention encompasses ceramidase and related nucleic acids, host cell expression systems, mutant ceramidase proteins, ceramidase fusion proteins, ceramidase antibodies, ceramidase antisense nucleic acids, and other compounds that modulate gene expression or ceramidase activity that can be used for prevention and treatment of proliferative disorders, including but not limited to breast cancer; cardiovascular diseases and inflammation.

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CERAMIDASE COMPOSITIONS AND METHODS BASED THEREON

This application claims priority to the provisional application no. 60/178,975, filed January 28, 2000, which is incorporated herein by reference in its entirety.

1. INTRODUCTION

The present invention relates to ceramidase genes, in particular to human mitochondrial ceramidase genes, and their encoded protein products, as well as derivatives and analogs thereof. The invention further relates to compositions and methods of diagnosis and therapy for diseases associated with cell overproliferation and sphingolipid signal transduction. In particular, the invention relates to the regulation of the level of ceramide by inhibiting ceramidase expression or activity.

2. BACKGROUND

2.1 CERAMIDE

Sphingolipid metabolites are now recognized as important components in signal transduction. Ceramide is one of these sphingolipid metabolites (Merrill, Jr., Nutr. Rev. 50:78 (1992), Kolesnick and Fuks, J. Exp. Med. 181:1949 (1995), Chao, Mol. Cell. Neurosci. 6:91 (1995), Liscovitch, Trends Biochem. Sci. 17:393 (1992). It has been shown to play a role in mediating, at least in part, the actions of these stimuli on cell differentiation, apoptosis, cell cycle arrest, and growth suppression. This is supported by the ability of exogenous analogs of ceramide to induce these biologic responses in the respective cell types (Hannun, 1994, J. Biol. Chem. 269:3125; Okazaki et al, 1990, J. Biol. Chem. 265:15823; Bielawska et al, 1992, FEBS Lett. 307:211; Obeid et al, 1993, Science 259:1769; Lauledérkind et al, 1995, J. Exp. Med. 182:599; Goldkorn et al, 1991, J. Biol. Chem. 266:16092; Perry and Hannun, 1998, Biochim. Biophys. Acta 1436:223-243; Mathias et al., 1998, Biochem. J. 335:465-480; and Dickson et al., 1999, Biochim. Biophys Acta. 1426:347-357). Furthermore, the action of a number of extracellular agents as well as stress stimuli, such as 1α,25-dihydroxyvitamin D_3, tumor necrosis factor α, interleukin-1β, neurotrophins, the Fas ligand, dexamethasone, serum withdrawal, chemotherapeutic agents, and γ-irradiation, can cause an elevation in the endogenous levels of ceramide (Hannun, J. Biol. Chem. 269:3125 (1994), Hannun and Obeid, Trends Biochem. Sci. 20:73

(1995), Ballou et al, J. Biol. Chem. 267:20044 (1992), Quintans et al, Biochem. Biophys. Res. Commun. 202:710 (1994), Dobrowsky et al, Science 265:1596 (1994), Yanaga and Watson, FEBS Lett. 314:297 (1992), Dressler and Kolesnick, Science 255:1715 (1992)).

Indeed, ceramide occupies a central position in sphingolipid metabolism. Complex sphingolipids can be derived from ceramide through various enzymatic reactions that add various head groups to the 1-hydroxyl position (Hannun, J. Biol. Chem. 269:3125 (1994), Wiegandt in Glycolipids (Weigandt. ed) pp. 199-259, Elsevier, New York (1985), Merrill, Jr. and Jones, Biochim. Biophys. Acta 1044:1 (1990), Van Echten and Sandhoff J. Biol. Chem. 268:53412 (1993), Hakomori, Annu. Rev. Biochem. 50:733 (1981)). The breakdown of these sphingolipids through sequential metabolic reactions also results in the formation of ceramide. In turn, ceramide can be degraded further through the action of ceramidases resulting in the formation of sphingosine and free fatty acids (Hannun, J. Biol. Chem. 269:3125 (1994), Spence et al, Biochem. Cell Biol. 64:400 (19867), Slife et al, J. Biol. Chem. 264:10371 (1989)).

Several mechanisms are involved in the regulation of cellular ceramide levels, which include activation of sphingomyelinases, activation of the *de novo* synthetic pathway, and inhibition of ceramidases (CDase). Ceramidases hydrolyze ceramide to form sphingosine, which in turn can serve as a substrate for sphingosine kinase, resulting in the formation of sphingosine1-phosphate. Ample evidence suggests distinct functions for these sphingolipids (Hannun et al., 2000, Trends Cell. Biol. 10: 73-80.)

2.2 CERAMIDASE

Ceramidases (CDases) are enzymes that cleave the N-acyl linkage of ceramide into sphingosine (SPH) and free fatty acid, and recent studies suggest that CDase may exert important functions in the regulation of its substrate (Cer) or in the regulation of its immediate product (SPH) or the downstream metabolite sphingosine 1-phosphate (SPP). Indeed, current understanding indicates that the major pathway for the formation of sphingosine is via the degradation of ceramide and not from the *de novo* pathway (Merill et al., 1992, Methods Enzymol. 209:427-437; Michel et al., 1997, J. Biol. Chem. 272:22432-22437.) This suggests that CDases are the key enzymes to regulate levels of SPH. Two reports implicate an alkaline CDase activity in signal transduction. Using cell homogenate of rat glomerular mesangial cells, Coroneos et al. have shown that an alkaline CDase activity was stimulated by the platelet-derived growth factor and not by the inflammatory

cytokines (tumor necrosis factor α and interleukin-1) or the vasoconstrictor peptide endothelin-1 (Coroneos et al., 1995, J. Biol. Chem. 270: 23305-23309.) In another report, Nikolova-Karakashian et al. showed in primary cultures of rat hepatocytes that alkaline CDase activity is stimulated by low concentrations of interleukin-1 (Nikolova-Karakashian et al., 1997, J. Biol. Chem. 272: 18718-18724.) The activation of CDase in these cells resulted in the formation of SPH, and these authors suggested that SPH or SPP may mediate some of the effects of low concentrations of interleukin-1.

Three ceramidases have been described thus far that differ by their pH optima. An acid ceramidase was first described by Gatt in rat brain (Gatt, 1963, J. Biol. Chem. 238: 3131-3133). The enzyme has been purified and cloned from human urine and recently from mouse tissue (Koch et al., 1996, J. Biol. Chem. 271:33110-33115; Li et al., 1998, Genomics 50: 267-274.) This enzyme is located in the lysosomes, and it plays a role in the catabolic pathway of ceramide, and the inherited deficiency of this enzyme causes Farber disease (Sugita et al., 1972, Science 178: 1100-1102.) A neutral activity has been described in liver plasma membranes and in rat intestinal brush border membranes; little is known about this enzyme (Slife et al., 1989, J. Biol. Chem. 264: 10371-10377; Nilsson et al., 1969, Biochim. Biophys. Acta 176: 339-347.) An alkaline activity was described in human cerebellum, fibroblasts, and in many rat tissues (Sugita et al., 1975, Biochim. Biophys. Acta 398: 125-131; Momoi et al., 1982, Biochem. J. 205: 419-425; Spence et al., 1985, Biochim. Cell Biol. 64: 400-404.) Alkaline CDases were best characterized in Guinea pig skin epidermis, where two enzymes were purified, one to apparent homogeneity and the other only partially (Yada et al., 1995, J. Biol. Chem. 270:12677-12684.) These two enzymes are membrane-bound, and their estimated molecular masses on SDS-PAGE were 60 and 148 ka, respectively.

Recent studies are also beginning to suggest a role for ceramidases in regulating the net levels of ceramide in response to stimuli. For example, it has been shown in rat hepatocytes that interleukin 1β at low concentration activates sphingomyelinases and ceramidases, resulting in the formation of sphingosine, whereas high concentrations of interleukin-1β, stimulated only sphingomyelinases resulting in the accumulation of ceramide (Nikolova-Karakashian et al., 1997, J. Biol. Chem. 272: 18718-18724.) In rat renal mesangial cells, both tumor necrosis factor α and nitric oxide donors have been shown to stimulate sphingomyelinases, but only nitric oxide donors inhibited ceramidases and resulted in an increase in ceramide levels and the consequent biological effects (Huwiler et

IN THE CLAIMS:

1. An isolated nucleic acid molecule comprising:

(a) the nucleotide sequence of SEQ ID NO: 1;

(b) a nucleotide sequence that encodes a polypeptide having the amino acid sequence of SEQ ID NO: 2;

(c) the nucleotide sequence of a ceramidase gene contained in plasmid Mito-CDase-TOPO/BII as deposited with the ATCC; or

(d) the complement of the nucleotide sequence of (a), (b) or (c).

2. An isolated nucleic acid molecule comprising a nucleotide sequence that hybridizes under medium stringent condition to a nucleic acid probe consisting of:

(a) the nucleotide sequence of nucleotide position 1 to nucleotide position 1702 or nucleotide position 2289 to nucleotide position 2583 of SEQ ID NO: 1;

(b) a nucleotide sequence that encodes a polypeptide having the amino acid sequence of amino acid position 1 to 554 or amino acid position 750 to amino acid position 761 of SEQ ID NO: 2; or

(c) the complement of the nucleotide sequence of (a), or (b);

wherein said medium stringent condition comprising prehybridization for 8 h to overnight at 55°C, in buffer composed of 6X SSC, 5x Denhart's with 0.5%SDS and 100 µg/ml salmon sperm DNA; hybridization for 48 h at 55°C in a buffer composed of 6X SSC, 5x Denhart's with 0.5%SDS and 100 µg/ml denatured salmon sperm DNA; and washing twice for 30 min at 60°C in a buffer composed of 1xSSC 0.1%SDS.

3. The isolated nucleic acid molecule of claim 1 or 2, which is genomic DNA, with the proviso that the isolated nucleic acid molecule does not consists of the nucleotide sequence of Genbank sequence accession no. AC 012131.

4. The isolated nucleic acid molecule of claim 1 or 2, which is cDNA.

5. The isolated nucleic acid molecule of claim 1 or 2, which is RNA.

6. An isolated nucleic acid comprising a nucleotide sequence that consists of at least 8

consecutive nucleotides of :

(a) the nucleotide sequence of nucleotide position 1 to nucleotide position 1702 or nucleotide position 2289 to nucleotide position 2583 of SEQ ID NO: 1;

(b) a nucleotide sequence that encodes a polypeptide having the amino acid sequence of amino acid position 1 to amino acid position 554 or amino acid position 750 to 761 of SEQ ID NO: 2; or

(c) the complement of the nucleotide sequence of (a), or (b).

7. A nucleic acid probe consisting of at least 8 nucleotides, wherein the nucleic acid probe is hybridizable under medium stringent condition to at least a portion of:

(a) the nucleotide sequence of nucleotide position 1 to nucleotide position 1702 or nucleotide position 2289 to nucleotide position 2583 of SEQ ID NO: 1;

(b) a nucleotide sequence that encodes a polypeptide having the amino acid sequence of amino acid position 1 to 554 or amino acid position 750 to amino acid position 761 of SEQ ID NO: 2; or

(c) the complement of the nucleotide sequence of (a) or (b);

wherein said medium stringent condition comprising prehybridization for 8 h to overnight at 55°C, in buffer composed of 6X SSC, 5x Denhart's with 0.5%SDS and 100 µg/ml salmon sperm DNA; hybridization for 48 h at 55°C in a buffer composed of 6X SSC, 5x Denhart's with 0.5%SDS and 100 µg/ml denatured salmon sperm DNA; and washing twice for 30 min at 60°C in a buffer composed of 1xSSC 0.1%SDS.

8. A nucleic acid molecule comprising the nucleotide sequence of a deletion mutant of :

(a) the nucleotide sequence of nucleotide position 1 to nucleotide position 1702 or nucleotide position 2289 to nucleotide position 2583 of SEQ ID NO: 1;

(b) a nucleotide sequence that encodes a polypeptide having the amino acid sequence of amino acid position 1 to 554 or amino acid position 750 to amino acid position 761 of SEQ ID NO: 2; or

(c) the complement of the nucleotide sequence of (a), or (b);

wherein said deletion mutant encodes a fragment of a ceramidase protein that displays one or more functional activities of ceramidase protein.

9. A nucleic acid molecule comprising the nucleotide sequence of a deletion mutant of :

(a) the nucleotide sequence of SEQ ID NO: 1;

(b) a nucleotide sequence that encodes a polypeptide having the amino acid sequence of SEQ ID NO: 2; or

(c) the complement of the nucleotide sequence of (a), or (b);

wherein said nucleotide sequence of said deletion mutant comprises more than 791 nucleotides.

10. The nucleic acid molecule of claim 8 or 9 in which the transmembrane domain, the signal peptide, the region of low compositional complexity, the phosphorylation site, the N-glycosylation site, or a combination thereof, are deleted.

11. The nucleic acid molecule of claim 8 or 9 in which one or more amino acid residues within the transmembrane domain, the signal peptide, the region of low compositional complexity, the phosphorylation site, or the N-glycosylation site, are deleted.

12. A nucleic acid molecule comprising a nucleotide sequence that hybridizes to the complement of the nucleic acid sequence of SEQ ID NO:1 and encodes a polypeptide with one or more activities of a ceramidase protein, linked uninterrupted by stop codons to a coding sequence that encodes a heterologous protein or peptide.

13. A nucleic acid molecule that is single-stranded, and that hybridizes under highly stringent conditions to a nucleic acid probe having the nucleotide sequence of SEQ ID NO:1.

14. A nucleic acid comprising a nucleotide sequence encoding a ceramidase-specific ribozyme, which comprises an autocatalytic cleaving ribozyme, and a region that hybridizes under highly stringent conditions to a nucleic acid having the nucleotide sequence of SEQ ID NO:1.

15. A recombinant vector comprising the nucleic acid molecule of Claim 1, 2, 6, 8, 9, or 12.

16. An expression construct comprising the nucleic acid molecule of Claim 1, 2, 6, 8, 9, or 12, wherein the nucleotide sequence is operatively associated with a regulatory nucleotide sequence containing transcriptional and/or translational regulatory signals that controls expression of the nucleotide sequence in a host cell.

17. A genetically engineered host cell containing the nucleic acid molecule of 1, 2, 6, 8, 9, or 12.

18. A genetically engineered host cell containing the nucleic acid molecule of 1, 2, 6, 8, 9, or 12, wherein the nucleotide sequence is operatively associated with a regulatory nucleotide sequence containing transcriptional and/or translational regulatory information that controls expression of the nucleotide sequence in the host cell.

19. A delivery complex comprising an expression construct comprising the nucleic acid molecule of Claim 1, wherein the nucleotide sequence is operatively associated with a regulatory nucleotide sequence containing transcriptional and/or translational regulatory signals that controls expression of the nucleotide sequence in a host cell, and a targeting means.

20. The delivery complex of claim 19, wherein the targeting means is selected from the group consisting of a sterol, a lipid, a virus, or a target cell specific binding agent.

21. A delivery complex comprising a nucleic acid molecule of Claim 13 or 14, and a targeting means.

22. The delivery complex of claim 21, wherein the targeting means is selected from the group consisting of a sterol, a lipid, a virus, or a target cell specific binding agent.

23. A transgenic non-human mammal in which the cells comprises a transgene encoding a ceradmidase protein having an amino acid sequence that is at least 60% identical to SEQ ID NO: 2, wherein the cells express the ceradmidase protein.

24. A transgenic non-human mammal whose somatic and germ cells comprise at least one genetically engineered disruption in a ceradmidase gene, wherein said ceradmidase gene hybridizes under medium stringency conditions to a nucleic acid molecule consisting of the nucleotide sequences of SEQ ID NO: 1, and wherein expression of ceradimase protein encoded by said ceradmidase gene is reduced; wherein said medium stringent condition comprising prehybridization for 8 h to overnight at 55°C, in buffer composed of 6X SSC, 5x Denhart's with 0.5%SDS and 100 µg/ml salmon sperm DNA; hybridization for 48 h at 55°C in a buffer composed of 6X SSC, 5x Denhart's with 0.5%SDS and 100 µg/ml denatured salmon sperm DNA; and washing twice for 30 min at 60°C in a buffer composed of 1xSSC 0.1%SDS.

25. A method for detecting in a sample the presence of a ceramidase nucleic acid, said method comprising:

(a) contacting the sample with a nucleic acid probe capable of hybridizing to at least a portion of the nucleic acid molecule of claim 1 under hybridizing conditions; and

(b) measuring the hybridization of the probe to the nucleic acids of the sample,

thereby detecting the presence of the ceramidase nucleic acid.

26. A method for detecting in a sample the presence of the ceramidase nucleic acid, said method comprising:

(a) contacting the sample with two diffferent nucleic acid primers capable of hybridizing to at least a portion of the nucleic acid molecule of claim 1 under hybridizing conditions;

(b) selectively amplifying the portion of the nucleic acid molecule of claim 1 flanked by the two nucleic acid primers; and

(c) detecting the amplified nucleic acid,

thereby detecting the presence of the ceramidase nucleic acid.

27. A method of making a ceramidase polypeptide comprising the steps of:

(a) culturing the cell of claim 18 under the appropriate conditions to produce ceramidase polypeptide; and

(b) isolating the ceramidase polypeptide.

28. An isolated ceramidase polypeptide comprising:

(a) an amino acid sequence encoded by the coding region of the nucleotide sequence of SEQ ID NO:1;

(b) the amino acid sequence of SEQ ID NO:2; or

(c) the amino acid sequence encoded by the coding region of a ceramidase gene contained in plasmid Mito-CDase-TOPO/BII as deposited with the ATCC.

29. A polypeptide encoded by the nucleic acid molecule of claim 2.

30. An isolated polypeptide, the amino acid sequence of which comprises at least six consecutive residues of SEQ ID NO: 2.

31. An isolated polypeptide, the amino acid sequence of which comprises at least one region selected from the group consisting of residues 1 to 19, 38-66, 176-196, 313-333, 431-451, 505-525, and 543-563 of SEQ ID NO: 2.

32. An isolated polypeptide, the amino acid sequence of which comprises SEQ ID NO: 2 with at least one conservative amino acid substitution.

33. An isolated polypeptide which is at least 60% identical to the ceramidase polypeptide having the amino acid sequence of SEQ ID NO:2, and displays one or more functional activities of ceramidase protein.

34. A chimeric protein comprising a fragment of a ceramidase protein consisting of at least 6 amino acids fused via a covalent bond to an amino acid sequence of a second polypeptide.

35. A pharmaceutical preparation comprising a therapeutically effective amount of the polypeptide of claim 28 and a pharmaceutically acceptable carrier.

36. An antibody preparation which binds a ceramidase protein of claim 28.

37. A molecule comprising a fragment of the antibody of claim 36, which fragment binds a ceramidase protein.

38. The antibody preparation of claim 36 which comprises a monoclonal antibody.

39. A method of diagnosing a disease or disorder characterized by an aberrant level of ceramidase RNA or protein in a subject, comprising measuring the level of ceramidase RNA or protein in a sample derived from the subject, in which an increase or decrease in the level of ceramidase RNA or protein, relative to the level of ceramidase RNA or protein found in an analogous sample not having the disease or disorder indicates the presence of the disease or disorder in the subject.

40. A method of diagnosing or screening for the presence of or a predisposition for developing a disease or disorder involving cell overproliferation or sphingolipid signal transduction in a subject comprising measuring the level of ceramidase protein, ceramidase RNA or ceramidase functional activity in a sample derived from the subject, in which a decrease in the level of ceramidase protein, ceramidase RNA, or ceramidase functional activity in the sample, relative to the level of ceramidase protein, ceramidase RNA, or ceramidase functional activity found in an analogous sample not having the disease or disorder or a predisposition for developing the disease or disorder, indicates the presence of the disease or disorder or a predisposition for developing the disease or disorder.

41. A method of diagnosing or screening for the presence of or a predisposition for developing a disease or disorder involving cell overproliferation sphingolipid signal transduction in a subject comprising detecting one or more mutations in ceramidase DNA, RNA or protein derived from the subject in which the presence of said one or more mutations indicates the presence of the disease or disorder or a predisposition for developing the disease or disorder.

42. A kit comprising in one or more containers a molecule selected from the group consisting of an anti-ceramidase antibody, a nucleic acid probe capable of hybridizing to a ceramidase RNA, or a pair of nucleic acid primers capable of priming amplification of at least a portion of a ceramidase nucleic acid.

43. A method of increasing the level of ceramide in a cell comprising contacting the cell with a compound that inhibits the ceramidase activity of the polypeptide of claim 28 in an amount sufficient to effect said inhibition.

44. A method of inhibiting the formation of sphingosine in a cell comprising contacting the cell with a compound that inhibits the ceramidase activity of the polypeptide of claim 28 in an amount sufficient to effect said inhibition.

45. A method of increasing the intracellular levels of ceramide in an animal comprising administering to said animal a compound that inhibits the ceramidase activity of the polypeptide of claim 28 in an amount sufficient to effect said inhibition.

46. A method of inhibiting the intracellular formation of sphingosine in an animal comprising administering to said animal a compound that inhibits the ceramidase activity of the polypeptide of claim 28 in an amount sufficient to effect said inhibition.

47. A method of treating a disease or disorder associated with cell overproliferation or sphingolipid signal transduction in an animal comprising administering to said animal a compound that inhibits the ceramidase activity of the polypeptide of claim 28 in an amount sufficient to effect said inhibition.

48. A method of treating a disease or disorder associated with cell overproliferation or sphingolipid signal transduction in an animal comprising administering to the animal an effective amount of the nucleic acid molecule of claim 13 that targets ceramidase transcripts, and interferes with translation of ceramidase transcripts.

49. A method of treating a disease or disorder associated with cell overproliferation or sphingolipid signal transduction in an animal comprising administering to the animal an effective amount of the nucleic acid molecule of claim 14 that targets ceramidase transcripts, and interferes with translation of ceramidase transcripts.

50. The method according to claim 47 in which the disease or disorder is selected from the group consisting of cancer, cardiovascular disorder, and inflammation.

51. The method according to claim 48 in which the disease or disorder is selected from the group consisting of cancer, cardiovascular disorder, and inflammation.

52. The method according to claim 49 in which the disease or disorder is selected from the group consisting of cancer, cardiovascular disorder, and inflammation.

53. A method of identifying a compound that binds to a ligand selected from the group consisting of a ceramidase protein, a fragment of a ceramidase protein comprising a domain of the protein, and a nucleic acid encoding the protein or fragment, comprising:

(a) contacting said ligand with a plurality of molecules under conditions conducive to binding between said ligand and the molecules; and

(b) identifying a molecule within said plurality that binds to said ligand.

54. A method for identifying compounds that modulate ceramidase gene expression comprising:

(a) contacting a test compound with a cell or cell lysate comprising an expression construct of claim 16; and

(b) detecting the transcription or translation of the nucleotide sequence of ceradmidase.

55. A method for identifying compounds that modulate ceramidase gene expression, comprising:

(a) contacting a test compound with a cell or cell lysate containing a reporter gene operatively associated with the regulatory element of a ceradmidase gene; and

(b) detecting expression of the reporter gene product.

56. A method for identifying compounds that modulate the activity of ceramidase gene product or homolog of ceramidase gene product comprising:

(a) contacting a test compound with an organism or a cell containing ceramidase gene product or homolog of ceramdiase; and

(b) comparing the phenotype of the organism or cell with the phenotype of organism or cell that did not contact the test compound, wherein a

change in phenotype indicates that the test compound is capable of modulating the activity of ceramidase gene product or homolog of ceramdiase gene product.

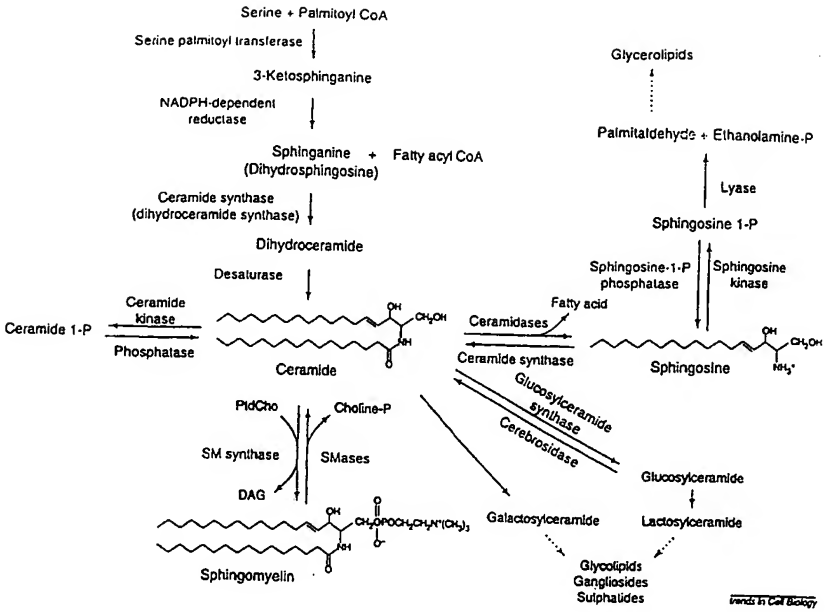

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FIG. 1